

Targeted analysis of polyphenol metabolism during development of hop (*Humulus lupulus* L.) cones following treatment with prohexadione-calcium

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Abstract:

Hops (*Humulus lupulus*), a main ingredient in beer, are valued as a source of bitter flavour and biologically active polyphenols. We treated immature hop cones with prohexadione-calcium (Pro-Ca), a flavanone-3-hydroxylase (F3H) inhibitor, to perturb the flavonoid pathway, and conducted a targeted analysis of 29 compounds from the phenolic acid, flavonoid, and terpenophenolic pathways, using photodiode array (UHPLC-PDA), and time of flight mass spectrometry (LC-TOF-MS) analysis. Hop cones were analysed over four stages of development spanning 22 days following Pro-Ca treatment. The content of terpenophenolics as well as metabolic precursors upstream from F3H significantly increased, and levels of flavonoid products downstream from F3H decreased. The methods developed here serve to compliment flavour analysis of polyphenol rich foods, and our results suggest ways to improve upon traditional agricultural methods to produce hops with altered chemical profiles.

Keywords: Flavonoids | *Humulus lupulus* | Phenolic acids | Prohexadione-calcium | Terpenophenolics

Article:

1. Introduction

Humulus lupulus L., hops, is a perennial vine that produces inflorescences, commonly known as “hop cones,” which contain economically and medicinally important compounds. Traditionally, hop cones have been valued for their high content of prenylated acylphloroglucinols, known as α - and β -acids, or humulones and lupulones, which have bittering flavours and antimicrobial properties that make hops a key ingredient in beer (Neve, 1991). More recently, a group of

biologically active prenylflavonoids, unique to hops, has been identified (Chen et al., 2012, Stevens et al., 1997, Zanolli and Zavatti, 2008).

In addition to prenylated compounds hops contain a number of polyphenols such as flavonoids and phenolic acids which have significant effects on flavour, stability, foaming, and process of brewed beverages (Aleksieva et al., 2005, Forster et al., 1995, Zhao et al., 2010). Beer is highly sensitive to oxidation and polyphenols, which exhibit antioxidant activity, can significantly increase the shelf life of products (Zhao et al., 2010). Moderate beer consumption can also significantly contribute to dietary polyphenols which may have effects at lowering the incidence of cardiovascular disease, diabetes, and some cancer types (Arranz et al., 2012).

Prenylated acylphloroglucinols and prenylflavonoids, collectively known as terpenophenolics, are biosynthetically produced via the phenolic acid, polyketide, and methylerythritol 4-phosphate (MEP) pathways (Fig. 1) (Page & Nagel, 2006). These compounds are produced in hop glandular trichomes; flavonoids, phenolic acids, and non-prenylated polyphenols, are found in the photosynthetic bract tissue of the hop inflorescence (Fig. 2) (Oliveira and Pais, 1990, Sugiyama et al., 2006). In hop bract flavonoid biosynthesis, malonyl-coenzyme A condenses with a single 4-coumaroyl-coenzyme A to produce naringenin chalcone, the precursor to naringenin. Naringenin can be hydroxylated by flavanone-3-hydroxylase (F3H) to produce polyphenols such as catechins, procyanidins, and flavonols, including kaempferol, quercetin, and their glycosides. In hop glandular trichomes, naringenin chalcone is prenylated by hop isoprenyl transferase 1 with the substrate dimethylallyl pyrophosphate (DMAPP) to produce prenylflavonoids (Fig. 1) (reviewed in Page, Liu, & Nagel, 2011). Biosynthesis of humulones and lupulones occurs by the condensation of malonyl-coenzyme A with acyl-coenzyme A, derived from branched chain amino acid degradation, for example isovaleryl-coenzyme A, the activated degradation product of valine (reviewed in Clark, Vaitheeswaran, Ambrose, Purves, & Page, 2013). This condensation reaction produces acylphloroglucinols, which are prenylated by aromatic prenyltransferases to produce humulones and lupulones.

Prohexadione-calcium (Pro-Ca) is a 2-oxoglutaric acid-dependent dioxygenase (2-ODD) inhibitor that has determined effects on critical enzymes involved in the production of gibberellins, ethylene, and flavonoids in apples (Rademacher et al., 1998, Zadravec et al., 2008). Pro-Ca has also been shown to have dramatic growth effects on hop plants when applied to immature stages at high doses (Kavalier et al., 2009), consistent with its agronomically favourable effects among several other valuable crops (Giudice et al., 2004, Miziniak and Praczyk, 2008, Xie et al., 2009, Zadravec et al., 2008). In addition to changes in growth and development, Pro-Ca has been shown to alter the production of flavonoids by inhibiting flavanone-3-hydroxylase (F3H), a 2-ODD essential to the biosynthesis of flavanols and procyanidins (Fig. 2) (Fischer et al., 2006, Gosch et al., 2003, Roemmelt et al., 2003). In response to Pro-Ca treatment, levels of phenolic acids and flavanones found upstream from F3H increased, while concentrations of compounds found downstream from F3H, including flavonols, flavan-3-ols, and procyanidins, significantly decreased in several species (Roemmelt et al., 2003). In addition to the alteration of known flavonoids, the presence of antimicrobial flavan-4-ols and 3-deoxyflavans were reported in several species, where they were previously unknown (Fig. 2) (Fischer et al., 2006, Gosch et al., 2003, Roemmelt et al., 2003).

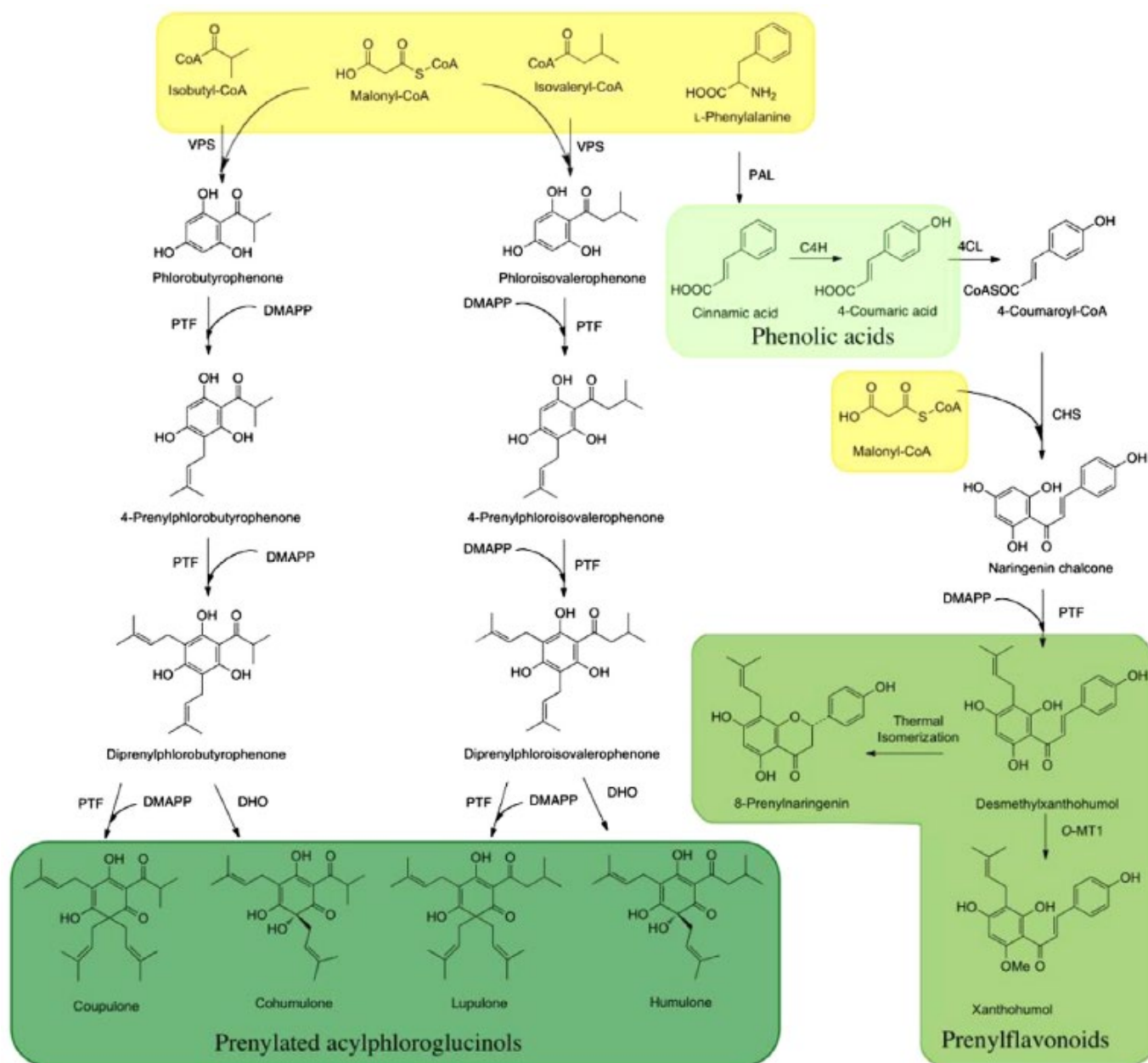
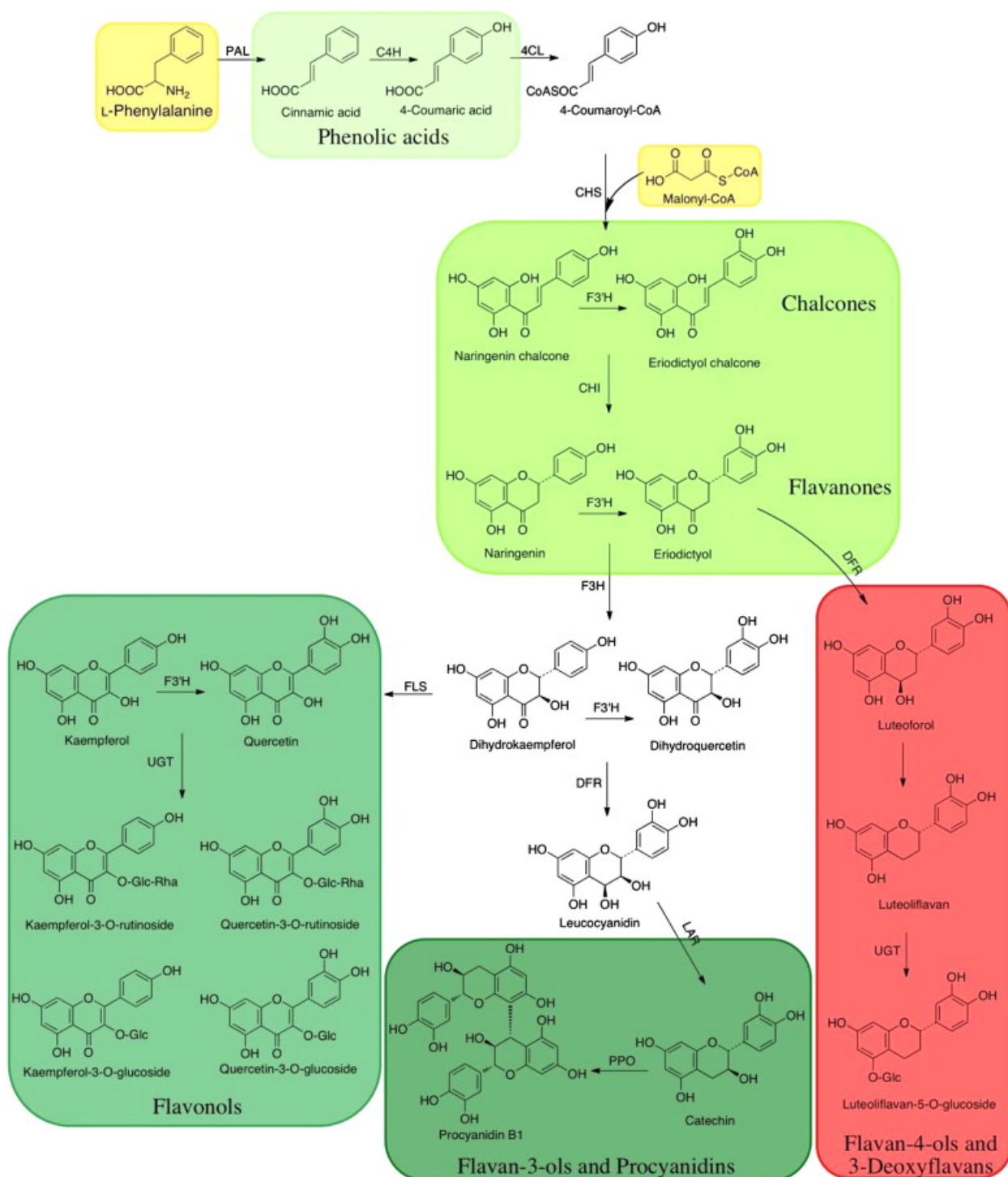


Fig. 1. Flavonoid and phenolic acid biosynthetic pathways. General pathways occurring in bracts and bracteoles of hop cones, including biosynthesis of flavan-4-ols and 3-deoxyflavans (red) which occurred in several crops following treatment with Pro-Ca. Primary metabolites are seen in yellow and groups containing phenolic acids and flavonoids quantitated in this study are various shades of green. Enzyme abbreviations are VPS: valerophenone synthase, PTF: aromatic prenyltransferase, DHO: deoxyhumulone oxidase, PAL: phenylalanine ammonia lyase, C4H: cinnamate 4-hydroxylase, 4-CL: coumarate coenzyme A ligase, CHS: chalcone synthase, O-MT1: *O*-methyl transferase 1, DMAPP: dimethylallyl diphosphate.



We have reported significant changes in accumulation of terpenophenolic contents of hop cones induced by Pro-Ca treatment; the direction and extent of change in terpenophenolic contents was dependent on the developmental stage of cones when the hop plants were treated (Kavalier et al., 2011). In order to gain a more accurate assessment of the metabolic changes induced by Pro-Ca treatment of hops, 29 polyphenolic compounds including precursors and products of the phenolic acid, flavonoid, and terpenophenolic pathways were quantitated from the “Zeus” cultivar at four developmental stages over 22 days following a single Pro-Ca treatment applied during early flower development.

2. Experimental

2.1. Plant material and treatment

Hop cultivar, *H. lupulus* ‘Zeus’, was grown and treated at Golden Gate Ranches, Hopsteiner-S.S. Steiner, Inc, near Prosser, WA. Plants were treated with 100 ppm Pro-Ca in conjunction with 1% (v/v) Regulaid™ (Kalo Inc., USA), a non-ionic surfactant, in deionized water. Control groups were treated with the surfactant in deionized water, and cones were collected at 2, 7, 15, and 22 days following treatment.

We have previously described five developmental stages of hop inflorescences, which were based on morphological and phytochemical properties of cones collected from near the plant apex (Kavalier et al., 2011). In the present experiment, we conducted Pro-Ca treatment on plants at what has been previously described as developmental stage I.

Ten biological replicates were collected for each group of five plants at each time-point; for each sample, ~10 g of fresh cones were collected near the apex of each bine. Samples were flash frozen in field using liquid nitrogen, then stored at –20 °C until extraction.

2.2. Extraction and UHPLC-PDA analysis of terpenophenolics

Hop cones were lyophilized, ground with liquid nitrogen, then extracted in 100% MeOH. Prenylflavonoids desmethylxanthohumol and xanthohumol, α -acids adhumulone, cohumulone, and humulone, and β -acids colupulone and lupulone were quantitated using a previously developed UHPLC-PDA method (Kavalier et al., 2009).

2.3. Extraction and sample preparation of polyphenols

After removing tissue for terpenophenolic analysis, samples were extracted by supercritical carbon dioxide and the resulting tissue was extracted in 80% MeOH using a previously described method (Kavalier et al., 2011).

2.4. HPLC-TOF-MS analysis of polyphenols

Twenty-two polyphenolic constituents were quantitated using a previously developed HPLC-TOF-MS method (Kavalier et al., 2011). Authentic phytochemical standards were used for all

phytochemical quantitation. MS data was collected in centroid mode using MassLynx V4.1 Scn 727 and processed using QuanLynx.

2.5. Statistical analysis

Statistical tests were conducted using JMP 8.01 (SAS, Cary, NC) software. Statistical significance was determined within a two-way ANOVA crossing treatment level (0 or 100 ppm Pro-Ca) with post treatment collection time-point (day 2, 7, 14, or 22). Significance was ascribed to differences among means where $p \leq 0.05$.

We also conducted multivariate statistical analysis using two-way MANOVA crossing treatment level (control or 100 ppm Pro-Ca) with the number of days following treatment that collections were made (days 2, 7, 15, or 22). Multivariate means were integrated for each of the seven terpenophenolics quantitated in this study, and we produced a scores plot with 95% confidence ellipses around the multivariate means using JMP 8.01 (SAS).

3. Results and discussion

Previous work has shown value-added changes in hop cone biomass production and terpenophenolic accumulation in hops resulting from Pro-Ca treatment (Kavalier et al., 2009, Kavalier et al., 2011). The substantial yield increases with low doses of Pro-Ca suggest the crop treatment is industrially relevant, but further work on chemical changes and substantial equivalence of the hop food products is necessary. We hypothesised that changes in terpenophenolics were likely paralleled by changes in flavonoids and phenolic acids, which have been altered in several crops following Pro-Ca treatment. Furthermore, due to the transient nature of Pro-Ca inhibition, we hypothesised that effects of treatment would change over time; therefore we assessed the effects of Pro-Ca treatment on terpenophenolics, phenolic acids, and flavonoids from hop cones collected at four developmental stages over a course of 22 days.

Careful developmental staging of cones allowed us to correlate several metabolite changes, which are partially concomitant with increased α - and β -acid accumulation (Kavalier et al., 2011). We quantitated seven major terpenophenolics including two prenylflavonoids (desmethylxanthohumol and xanthohumol), three α -acids (adhumulone, cohumulone, and humulone), and two β -acids (colupulone and lupulone) (Kavalier et al., 2009). In addition to terpenophenolics, we quantitated polyphenolic constituents present in photosynthetic bract tissue: three subclasses of flavonoid metabolites found biosynthetically downstream from F3H, (1) flavan-3-ols (catechin and epicatechin), (2) procyanidins (B-1 and B-2), and (3) flavonols (kaempferol and quercetin) and flavonol glycosides (kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-rutinoside). Five subclasses of metabolites found biosynthetically upstream from F3H were also measured, (1) a chalcone (naringenin chalcone), (2) a flavanone (naringenin), (3) phenolic acids (chlorogenic acid, 4-coumaric acid, ferulic acid, and 4-hydroxybenzoic acid), (4) a stilbene (resveratrol), and (5) three amino acids (phenylalanine, leucine, and valine), many of which are precursors in the biosynthesis of the flavonoids and polyketides of interest (Fig. 1, Fig. 2). Synthesis and storage of these different metabolite classes are compartmentalised in different cells; polyphenols and phenolic acids are found in mesophyll and epidermal cells whereas

terpenophenolics are present in trichome secretory cells (Oliveira and Pais, 1990, Sugiyama et al., 2006).

After treatment with Pro-Ca, significant changes in the content of 24 of the 29 compounds were measured as compared to untreated controls, where steady-state accumulations were quantitated at four time-points over 22 days following treatment of hop plants with Pro-Ca. Compounds within the same structural classes changed in quantity with similar trends in respect to the direction, extent, and timing of changes in treated samples as compared to controls. Increases in terpenophenolics, present in glandular trichomes, were accompanied by changes in the contents of flavonoids, chalcones, and phenolic acids found in the cone bract tissue.

3.1. Changes in accumulation of terpenophenolics

Terpenophenolic contents of mature Zeus cultivar hop cones significantly increased following treatment at the middle and late stages of flowering. However, when Zeus plants were treated at an early stage of development, significant decreases in terpenophenolics were measured by the time of harvest, when hop cones had reached full maturity. Here, we treated hop plants at an early stage of development and monitored accumulation of terpenophenolics in the days immediately following treatment in order to investigate developmental changes over a wider time-course.

Prenylflavonoid contents increased on day 15 following treatment, then returned to levels found in controls by day 22 after treatment (Fig. 3a). No changes in α - or β -acids were observed through day 15; on day 22 following treatment all five compounds increased (Fig. 3b). Co-multifidol glycoside, a glycosylated prenylated acylphloroglucinol, also increased on day 22 after treatment, but this trend was not statistically significant at 95% confidence. With the exception of co-multifidol glycoside, all prenylated compounds studied showed minor decreases 2 days after Pro-Ca treatment, but none of these decreases were statistically significant.

We compared the seven terpenophenolics quantitated by two-way MANOVA and graphed the results with 95% confidence ellipses (Fig. 4). Results from the MANOVA revealed significant differences between control and treatment samples when collected at day 2 following treatment. Additionally, treatment samples collected at day 15 following Pro-Ca application significantly differed from all other groups as this group was separated from other clusters by contents of xanthohumol and humulone. Two-way ANOVA showed significant increases in levels of xanthohumol and desmethylxanthohumol in treatment samples collected on day 15 following treatment; however, levels of α - and β -acids were not significantly different from controls. In the MANOVA, treatment samples collected at day 22 following Pro-Ca application formed a cluster that significantly differed from all other groups. These results matched those found by two-way ANOVA where α - and β -acid contents significantly increased in the treatment group. Changes in treatment samples collected at day 22 following Pro-Ca application clustered due to colupulone and lupulone contents, and to a lesser extent the clustering was also due to adhumulone content. Overall, the two-way MANOVA produced five separate clusters as shown by non-overlapping 95% confidence ellipses of the multivariate means (Fig. 4).

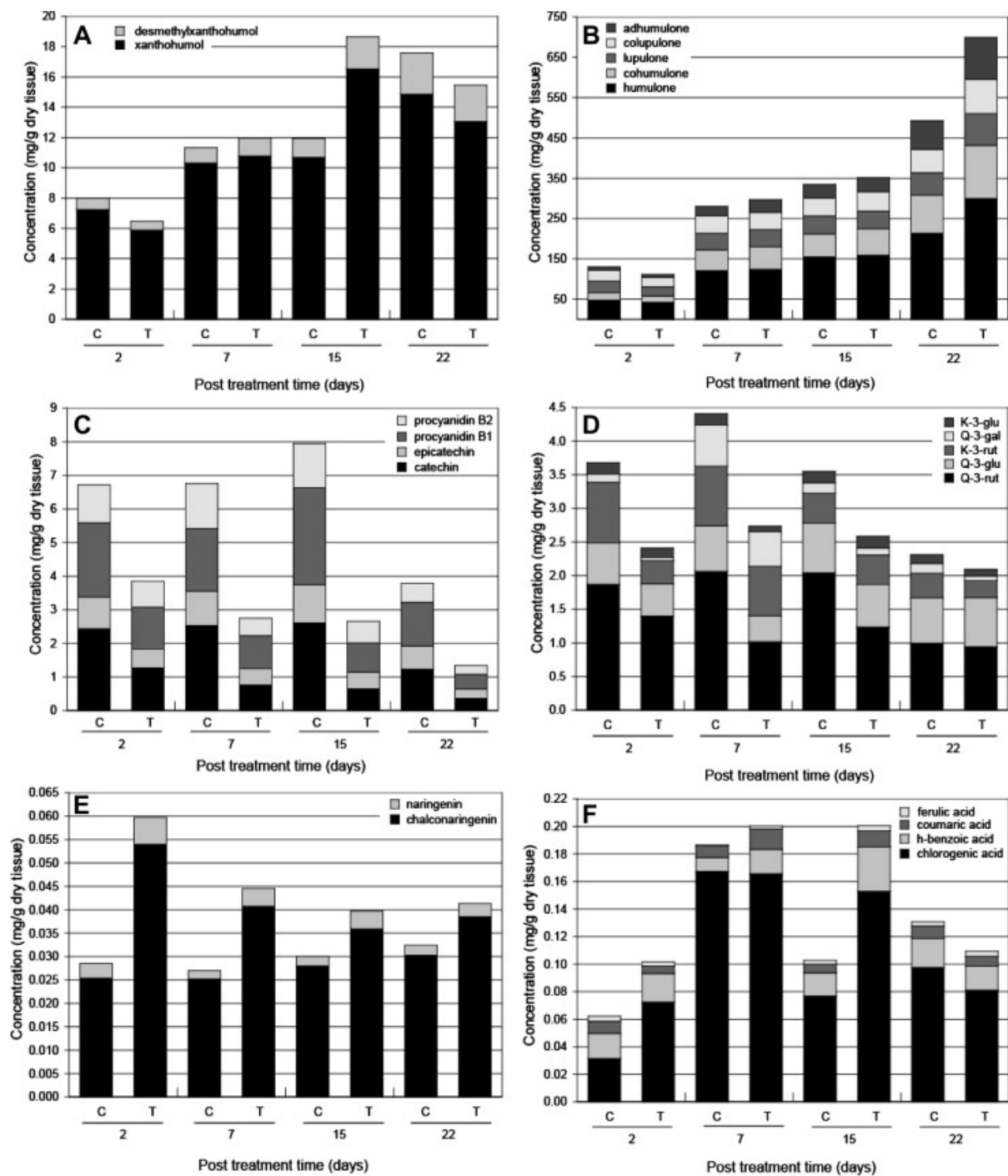


Fig. 3. Phytochemical changes induced by Pro-Ca treatment. Effects of Pro-Ca on (A) prenylflavonoids xanthohumol and desmethylxanthohumol, (B) α - acids adhumulone, cohumulone, humulone and β -acids colupulone and lupulone, (C) flavan-3-ols and procyanidins, and (D) flavonol glycosides, (E) naringenin chalcone and naringenin and (F) phenolic acids chlorogenic acid, 4-hydroxybenzoic acid, 4-coumaric acid and ferulic acid. Control (C) and Pro-Ca treated (T) were quantitated on days 2, 7, 15, and 22 following Pro-Ca application. Compound abbreviations are K-3-glu: kaempferol-3-*O*-glucoside, Q-3-gal: quercetin-3-*O*-galactoside, K-3-rut: kaempferol-3-*O*-rutoside, Q-3-glu: quercetin-3-*O*-glucoside, Q-3-rut: quercetin-3-*O*-rutoside, and h-benzoic acid: 4-hydroxybenzoic acid.

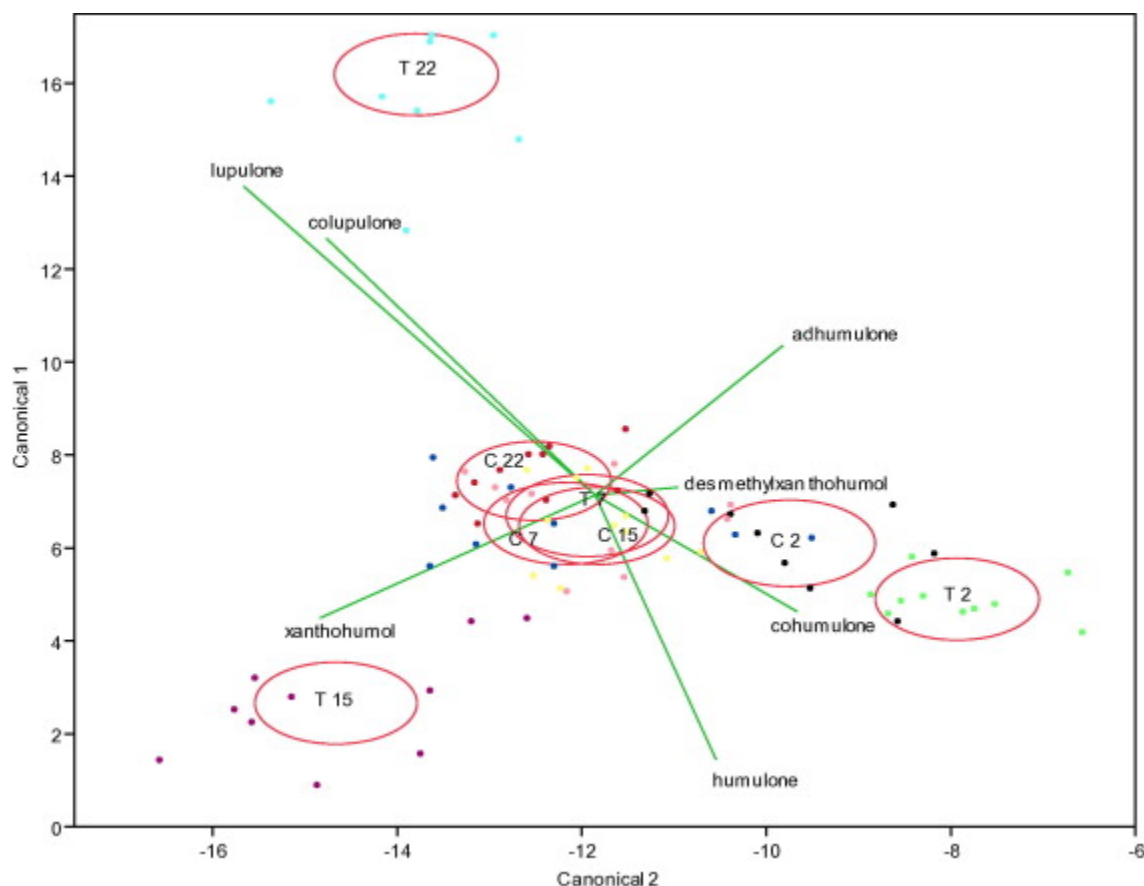


Fig. 4. Score plot from a two-way MANOVA with 95% confidence ellipses. Loading vectors are shown in green and labelled as each of seven terpenophenolics. Abbreviations for each group are C: control, T: treatment, numbers correspond to number of days between treatment and collection. The score plot shows individual samples with colors black: C2, green: T2, dark blue: C7, yellow: T7, pink: C15, purple: T15, red: C22, light blue: T22. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Loading plot vectors indicate a difference among humulones and lupulones and xanthohumol in separation of the clusters by time-after-treatment. While these secondary metabolites differ in metabolic precursors, they also noticeably differ in the number of prenylations, i.e., the lupulones, which are uniquely prenylated three times by an aromatic prenyl transferase activities, have similar vectors. These results suggest specific effects of Pro-Ca on the metabolism of trichomatous glands, either by modulation of timing of development of the glands or modulation of precursor pools over development of the glands.

In order to further characterise the nature of the changes in metabolism, we conducted linear regression analysis on the content of seven terpenophenolics including desmethylxanthohumol, xanthohumol, cohumulone, humulone, adhumulone, colupulone, and lupulone. By graphing the accumulation of these compounds over five time-points following treatment with 100 ppm Pro-Ca, we noted significant changes in the velocity of accumulation of α - and β -acids (Fig. 5 and Table 1). The slope of the linear fit for each component shows the average increase in compound yield per day as measured in mg of each compound/g dried hop cone weight.

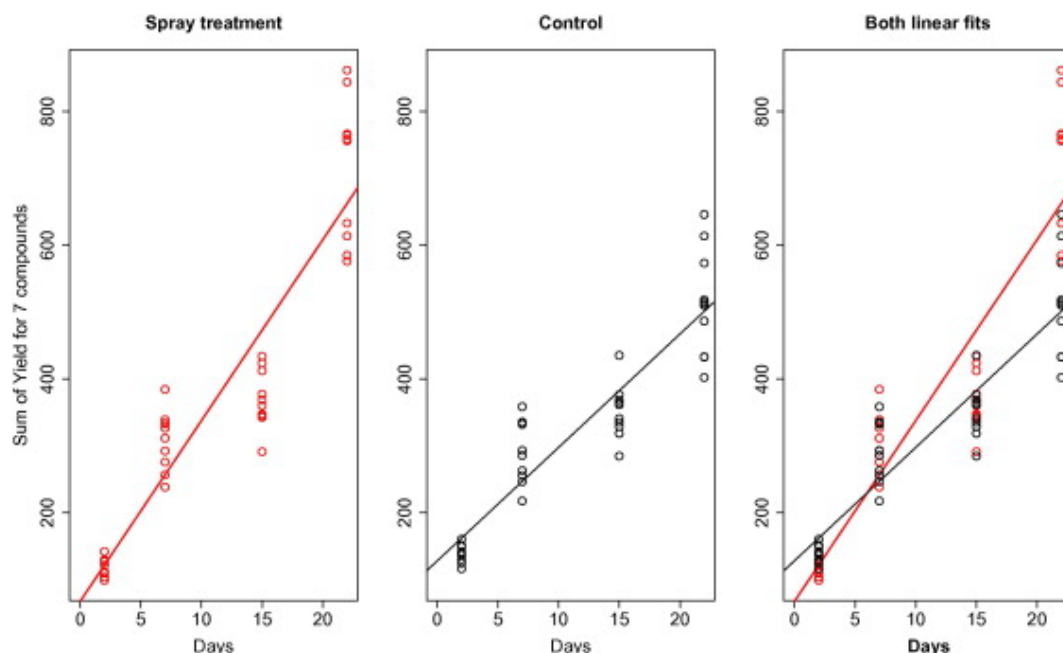


Fig. 5. Linear regression analysis for the sum of seven terpenophenolic compounds from treatment (red) and control (black) Zeus hop cones collected on days 2, 7, 15, and 22 following treatment with 100 ppm Pro-Ca. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1. Least squares linear regression analysis of the time course (day 2, 5, 15, 22) of compound yield in control versus Pro-Ca treated (spray treatment) hop plants. The slope of the fitted line reflects average increase in yield per day.

Treatment					Control					
Compound	Slope	N	<i>p</i> -Value ^a	R-sq.%	95% CI ^a	Slope	N	<i>p</i> -Value ^a	R-sq.%	95% CI ^a
Humulone	11.802	40	<0.0001	80.2	9.88, 13.72	7.612	41	<0.0001	76.6	6.25, 8.98
Cohumulone	5.228	40	<0.0001	81.4	4.41, 6.05	3.43	41	<0.0001	78.8	2.85, 4.01
Adhumulone	4.33	40	<0.0001	77.9	3.57, 5.09	2.946	41	<0.0001	83.1	2.52, 3.38
Colupulone	2.773	40	<0.0001	78.1	2.26, 3.21	1.375	41	<0.0001	71.6	1.09, 1.66
Lupulone	2.455	40	<0.0001	79.2	2.04, 2.87	1.153	41	<0.0001	72.9	0.93, 1.38
Xanthohumol	0.388	40	<0.0001	52.7	0.27, 0.51	0.338	41	<0.0001	71.7	0.27, 0.41
Desmethxyl-xanthohumol	0.094	40	<0.0001	82.5	0.08, 0.11	0.0935	41	<0.0001	73.1	0.08, 0.11
Sum	27.028	40	<0.0001	85.1	23.3, 30.7	16.947	41	<0.0001	83.4	14.5, 19.4
Heterogeneity of slope (treatment vs. control)					Heterogeneity of fitted line (treatment vs. control)					
Compound	Difference	<i>p</i> -Value ^b	95% CI ^c	<i>F</i>	df	<i>p</i> -Value				
Humulone	4.190	0.00071	1.87, 6.40	9.94	2.77	0.00014				
Cohumulone	1.798	0.0005	0.97, 2.58	11.83	2.77	<0.0001				
Adhumulone	1.383	0.0022	0.71, 2.02	10.40	2.77	0.0001				
Colupulone	1.358	<0.0001	0.89, 1.81	17.90	2.77	<0.0001				
Lupulone	1.302	<0.0001	0.95, 1.62	19.20	2.77	<0.0001				
Xanthohumol	0.050	0.47	−0.02, 0.12	1.45	2.77	0.24				
Desmethxyl-xanthohumol	0.0005	0.97	−0.19, 0.02	1.61	2.77	0.21				
Sum	10.082	<0.0001	6.46, 13.62	16.70	2.77	<0.0001				

^a Calculated in the traditional manner using the *t*-distribution.

^b Permutation test with 100,000 randomizations, two-sided.

^c Stratified bootstrap with 100,000 samples in each treatment-time combination.

The linear regression plot of the sum of all seven terpenophenolics from treatment vs. control shows a significant increase in the velocity of accumulation of terpenophenolics in treatment cones (red) as compared with controls (black) (Fig. 5). Linear regression values are provided for each compound from control and treatment cones in Table 1. Analysis of the homogeneity of the slope of each compound's linear fit shows significant increases in the velocity of accumulation of each of the five α - and β -acids for treatment vs. control, with p-values ranging from 0.0022 to <0.001 . Significant changes in the rate of accumulation of prenylflavonoids, desmethylxanthohumol and xanthohumol, however, did not occur. Increase in terpenophenolic accumulation in response to Pro-Ca treatment may be due to a number of causes that are not mutually exclusive. Hypothetical causes include: (1) increase in trichome number and size, (2) increase in trichome secretory cell metabolic rate, (3) increase in primary metabolic precursor pools to terpenophenolics, or (4) protraction of glandular trichome development resulting in more accumulation time (Kavalier et al., 2011). Increased accumulation velocities alone may be sufficient to cause increased secondary metabolite accumulation in trichomatous glands. These increased velocities theoretically allow for the possibility that protracted accumulation time is unnecessary to enhanced secondary metabolite accumulation. Therefore, increase growth rate of glands or increase metabolic flux may account for the observed yield gains. Our finding of velocity increases allows crucial experiments to be designed to delineate the causes. The observation that velocity increase occurred for bitter acids, but not prenylflavonoids, suggests a metabolic cause for induced increases in secondary metabolite accumulation, rather than a gross change in gland number or gland size. Nevertheless, since the timing of maximal accumulation of prenylflavonoids and bitter acids are different (Kavalier et al., 2011) both metabolite precursor pools and subtle growth effects could differently effect accumulation rates.

3.2. Changes in accumulation of metabolites downstream from F3H

Flavonoids are secondary metabolites resulting from a linear array of pathways, thus, blockage of the early pathway enzymes by inhibitors leads to decreased contents of the secondary metabolites. A homeostatic network of metabolism (or complex catabolism) is not expected for flavonoid accumulation in terminal, reproductive organs. Reactivation of stored glycosides to aglycones in response to stress is well-documented (Treutter, 2006), but is not likely to be a major contributor to content distribution in our agronomically-regulated field environment: no major water stress, herbivory, or insect damage was observed during our studies.

Since flavan-3-ols, procyanidins, flavanols, and flavanol glycosides are products derived from dihydroflavanols, and are the terminal products of the hydroxylation of flavanones catalysed by F3H (Fig. 2), we hypothesised decreased contents of these compounds in response to F3H inhibition. Dihydroflavanols can be oxidised by flavanol synthase (FLS) to produce flavanols; alternatively, dihydroflavanols can be reduced by dihydroflavonol reductase (DFR) to produce flavan-3,4-ols, which can be further reduced by leucoanthocyanidin reductase (LAR) to produce flavan-3-ols. Flavan-3-ols can also form polymeric compounds known as procyanidins. Procyanidins are important to flavour of hops and the processing of beer, and potentially, to the ecology of the hop crop, as they are antioxidants and anti-herbivory compounds (Deinzer, 2006, Li and Deinzer, 2009). The effects of Pro-Ca on accumulation of compounds found downstream from F3H were assessed by the quantitation of flavan-3-ols, procyanidins, flavanols,

and flavanol glycosides. Dihydroflavanols and leucocyanidins were not present in crude extracts above the limit of quantitation and were therefore not analysed in this study.

Flavan-3-ols, catechin and epicatechin, significantly decreased in cones collected 2, 7, and 15 days following treatment, with the exception of 2 days following treatment when the decreases in epicatechin were not statistically significant (Fig. 3c). Similar to flavan-3-ols, procyanidins B1 and B2, dimers of catechin and epicatechin, decreased in cones from treated plants throughout the experiment but only significantly decreased on days 7 and 15 following treatment (Fig. 3c). Significant changes in both flavan-3-ols and procyanidins reflect a large and rapid decrease in contents of metabolites present downstream from F3H in response to Pro-Ca treatment.

Flavonols quercetin, kaempferol, and their respective glycosides also significantly decreased following Pro-Ca treatment (Fig. 3d). Quercetin levels decreased on days 15 and 22 following treatment, and kaempferol contents decreased 2 days after treatment. All quercetin glycosides measured, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, and quercetin-3-*O*-glucoside, significantly decreased by day 7 after treatment, additionally quercetin-3-*O*-rutinoside levels were significantly lower in treatment plants on day 15 following Pro-Ca application. Kaempferol glycosides, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside, significantly decreased 2 days following treatment. Levels of flavonol glycosides had similar trends in their responses to Pro-Ca treatment as their respective aglycones. Kaempferol, kaempferol-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside levels all significantly decreased on day 2 following treatment. Similar to kaempferol and kaempferol glycosides, levels of quercetin and quercetin glycosides shared the same trend in content changes in response to Pro-Ca treatment; quercetin content did not significantly decrease until 15 days after treatment and quercetin glycoside levels significantly decreased by day 7 following treatment.

The timing of accumulation of quercetin and quercetin glycosides in comparison to kaempferol and kaempferol glycosides may be related to activity of F3'H on flavonol hydroxylation. Quercetin and kaempferol differed in the extent of hydroxylation on the flavonoid B ring (Fig. 1). Kaempferol has a single OH group on ring B attached at the 4' carbon; whereas quercetin contains two OH groups attached at carbons 3' and 4'. Kaempferol may be hydroxylated on the 3' carbon by F3'H to produce quercetin. Naringenin and dihydrokaempferol, the 4'-hydroxylated precursors to kaempferol, were present in the 80% MeOH extract at detectable levels by LC-TOF-MS, while eriodictyol and dihydroquercetin, the 3',4'-hydroxylated precursors to quercetin, were not. Quercetin and quercetin glycosides, however, are present at greater levels than kaempferol and kaempferol glycosides in hop cones. It is therefore likely that F3'H activity is responsible for the accumulation of quercetin in hop cones, and the additional time between Pro-Ca treatment and the responses of quercetin and quercetin glycoside levels, as compared to levels of kaempferol and kaempferol glycosides, may be due to the F3'H catalysed hydroxylation. Additionally, F3'H is a 2-ODD that may also be inhibited by Pro-Ca. Flavonol production may be further inhibited at flavonol synthase (FLS), which is also a 2-ODD that has been shown to be inhibited by Pro-Ca treatment (Roemmelt et al., 2003). In summary, while varying in the timing and extent of changes, levels of flavan-3-ols, procyanidins, flavonols, and flavonol glycosides, all biosynthetically produced downstream from F3H, significantly decreased following Pro-Ca

treatment (Fig. 3c and d). These changes support our hypothesis that Pro-Ca inhibits F3H in hop cones.

3.3. Changes in accumulation of metabolites upstream from F3H

Although previous results from apple trees treated with Pro-Ca showed significant increases in eriodictyol, a 3',4'-hydroxylated flavanone (Roemmelt et al., 2003), we were interested in levels of prenylflavonoid precursor naringenin chalcone, the precursor of naringenin, a 4' hydroxylated flavanone. While hops contain significant levels of naringenin and naringenin chalcone, eriodictyol and eriodictyol chalcone have not been reported in the plant, and we were unable to detect these compounds by TOF-MS. Naringenin and naringenin chalcone contents doubled two days following Pro-Ca treatment and slowly returned to values found in controls over 22 days (Fig. 3e). Naringenin contents were significantly greater in treated cones on days 2, 7, and 15 following treatment, and naringenin chalcone levels were significantly greater on days 2 and 7 following treatment. While chalcones have not previously been studied in Pro-Ca experiments, the increases in naringenin we measured in response to treatment parallel previous findings in which flavanones and flavanone glycosides accumulated following Pro-Ca treatment in several crops (Fischer et al., 2006, Gosch et al., 2003, Roemmelt et al., 2003).

Ferulic, 4-hydroxybenzoic, 4-coumaric, and chlorogenic acids were measured to gauge the changes in steady-state accumulations of phenolic acids to Pro-Ca treatment. These compounds are significant as ferulic, chlorogenic, and 4-hydroxybenzoic acids are all produced from 4-coumaric acid, which is a precursor compound in naringenin chalcone synthesis. Levels of all phenolic acids measured significantly increased following Pro-Ca treatment (Fig. 3f). Coumaric acid and 4-hydroxybenzoic acid levels significantly increased on days 7 and 15 after treatment. Ferulic acid contents significantly increased 7 days after treatment, and chlorogenic acid levels significantly increased on day 15 following treatment. Contents of all four phenolic acids in treated cones returned to levels found in controls by day 22 following treatment. Our results show metabolic changes in phenolic acids that are present several biosynthetic steps upstream from F3H (Fischer et al., 2006, Gosch et al., 2003, Roemmelt et al., 2003).

Coumaric acid and naringenin chalcone are precursor compounds to prenylflavonoid biosynthesis (Maloukh, 2010, Nagel et al., 2008, Page and Nagel, 2006). However, because of organ-level compartmentalisation, a direct correlation between the increases in these precursor compounds and the accumulation of terpenophenolics is not likely. The inhibition of F3H by Pro-Ca treatment has previously shown to increase flavanones and phenolic acids in vegetative tissues; based on previous studies we hypothesise that the effects on phenolic acids and naringenin chalcone is likely occurring in the vegetative bract tissue of hop cones.

Terpenophenolics accumulate in the glandular trichomes present on the abaxial surface of bracteoles present in hop cones, whereas flavonoid and phenolic acid constituents are found in the leafy bract tissue (Sugiyama et al., 2006). Because of the compartmentalisation of the secondary metabolites, partitioning is likely to occur at the level of primary metabolism, especially by influences on the carbohydrate and amino acid pools that are shared across different organs of the inflorescences (Fischer et al., 1998).

Despite increases in terpenophenolic accumulation, we did not detect changes in the levels of phenylalanine, leucine, or valine after Pro-Ca treatment. While, phenylalanine is an aromatic precursor to phenolic acid, flavonoid, and prenylflavonoid production, branch-chain amino acids are the metabolic precursors of hop bitter acids. For example, leucine, from which the activated degradation product iso-valeryl-CoA is produced, is a precursor for humulone and lupulone production. Likewise, valine is the precursor for isobutyryl-CoA, used in cohumulone and colupulone production (Fig. 1). While terpenophenolic production does provide a significant sink for phenylalanine and the degradation products of leucine and valine, these substrates are used in multiple cellular processes and their relative contents are likely the result of multiple sink allocations, replenishment from branch-chain amino acid metabolism, and transport. We have previously discussed the potential inhibitory effects of Pro-Ca on amino transferases, but our results do not indicate inhibition (Kavalier et al., 2011). Overall, we measured significant increases in several metabolites found upstream from F3H in the flavonoid and phenolic acid pathways following treatment of hop plants with Pro-Ca (Fig. 3e and f).

In summary, following Pro-Ca treatment of hop plants during early cone development, cones accumulated higher levels of terpenophenolics than untreated hops; increases in phenolic acids, naringenin chalcone, and naringenin also occurred. Increased production of these compounds, located biosynthetically upstream from F3H, coincided with significant decreases in catechins, procyanidins, flavonols, and flavonol glycosides, which are found biosynthetically downstream from F3H in the flavonoid pathway. In most cases, steady-state contents of compounds found downstream from F3H rapidly decrease in content in response to Pro-Ca treatment, which occurred on days two and seven following treatment. As the season progressed compounds in treated cones returned to levels found in controls. This is likely due to the metabolism of Pro-Ca within the plant; Pro-Ca has a short half-life of a few weeks and is rapidly broken down into endogenous plant metabolites.

Other studies of metabolic perturbation induced by Pro-Ca treatment including effects on flavonoid and phenolic acid biosynthesis have been published, but no other reports of Pro-Ca effects on glandular trichome secondary metabolite accumulation over time have been reported. Importantly, we show for the first time that velocity of accumulation of glandular secondary metabolite accumulation could be manipulated with an antimetabolite. The causal components of the velocity change may now be delineated by a crucial experiment, by measuring either gland density and growth or metabolic flux. Our results parallel those found in leaves of apple trees following treatment with Pro-Ca and add additional insight into changes of phenolic acids, flavonoids, and hop-specific terpenophenolic metabolism induced by Pro-Ca. Previously, analysis of metabolic effects of Pro-Ca on the phenolic acid and flavonoid pathways in apple included quantitation of 12 components found throughout these pathways (Roemmelt et al., 2003). In this study we developed a more comprehensive targeted analysis and have built on previous studies which suggest the value of Pro-Ca for crop improvement in hops. Hops treated with Pro-Ca exhibit changes in phytochemical content, many of which seem not to extend throughout maturity. Changes in chemical content are consistent with other Pro-Ca modified agricultural products, which have been met with deregulatory approval and widespread production. Larger-scale agronomics experiments are proposed, and further application knowledge for successful seasonal, varietal and climatic variants in treatment is imminent.

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